EXHIBIT 2

Improved Induction of Melanoma-Reactive CTL with Peptides from the Melanoma Antigen gp100 Modified at HLA-A*0201-Binding Residues

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Recognition of the melanoma Ag gp100 by tumor-infiltrating lymphocytes (TIL) in vitro has been correlated with tumor regression in patients with metastatic melanoma treated with the adoptive transfer of TIL plus IL-2. Three common gp100 epitopes have been identified that are recognized in the context of HLA-A2 by TIL from different patients: $G9_{154}$ (KTWGQYWQV), $G9_{209}$ (ITDQVPFSV), and $G9_{280}$ (YLEPGPVTA). Upon stimulation with these peptides, melanoma-reactive CTL could be induced in vitro from PBL of some HLA-A2$^+$ melanoma patients. However, numerous restimulations were required, and specific reactivity could not be generated in many patients. Therefore, to enhance the immunogenicity of gp100 peptides, amino acid substitutions were introduced into $G9_{154}$, $G9_{209}$, and $G9_{280}$ at HLA-A*0201-binding anchor positions, but not at TCR contact residues, to increase peptide class I MHC-binding affinity. Several modified gp100 peptides bound with greater affinity to HLA-A*0201 than unmodified peptides and were recognized by TIL specific for the natural epitopes. These peptides were used to sensitize PBL from HLA-A2$^+$ melanoma patients in vitro using peptide-pulsed autologous PBMC as stimulators. After five weekly restimulations with either the native $G9_{209}$ or $G9_{280}$ peptide, melanoma-reactive CTL could only be induced from two of seven patients. However, amino acid substitutions in these peptides enabled the induction of melanoma-reactive CTL from all seven patients. These results suggest that modified gp100 peptides may be more immunogenic than the native epitopes, and may be useful in immunotherapy protocols for patients with melanoma. *The Journal of Immunology,* **1996, 157: 2539–2548.**

Clinical evidence strongly suggests that T lymphocytes play a significant role in the regression of melanoma in vivo. In a previous clinical investigation, the adoptive transfer of cultured T cells from tumor-infiltrating lymphocytes (TIL)[2] with IL-2 resulted in tumor regression (complete and partial responses) in 30 to 40% of patients with metastatic melanoma (1–3). In vitro many TIL recognize autologous melanoma cells, as well as allogeneic melanomas and melanocytes that share particular class I MHC molecules (4–9). In addition, six Ags have been identified that are recognized by TIL that mediated tumor regression when transferred adoptively into autologous patients with IL-2: four melanosomal proteins (MART-1 (10, 11), gp100 (12, 13), tyrosinase (14, 15), and TRP-1 (16)), and two other proteins (p15 (17) and β catenin (18)). Therefore, class I MHC-restricted recognition of epitopes from these Ags may be involved in mediating tumor rejection.

A significant correlation between T cell recognition in vitro and tumor regression in patients receiving TIL therapy has been demonstrated for the HLA-A2-restricted melanoma Ag, gp100 (19), suggesting that this protein may be a potent tumor rejection Ag. gp100 is a melanocyte lineage-specific membrane glycoprotein consisting of 661 amino acids, and is expressed in most melanoma cells (13). This protein is recognized by 8 of approximately 20 HLA-A2-restricted melanoma-reactive TIL established in our laboratory. Five T cell epitopes have been identified in gp100, and three of these are common immunogenic epitopes recognized by TIL derived from different patients (19–21): $G9_{154}$ (KTWGQYWQV), $G9_{209}$ (ITDQVPFSV), and $G9_{280}$ (YLEPGPVTA). As a preliminary evaluation of the use of gp100 peptides in melanoma vaccine strategies, several of these peptides were used to induce CTL in vitro from PBL of HLA-A2$^+$ melanoma patients (22). Melanoma reactivity was observed by T cells sensitized with four gp100 peptides; however, with a given peptide, melanoma-reactive CTL could only be induced in a limited number of patients, and numerous restimulations of responding lymphocytes were required to generate melanoma reactivity. These findings prompted the current investigation aimed at enhancing the immunogenicity of peptides derived from gp100.

A correlation has been demonstrated between immunogenicity and peptide-binding affinity to class I MHC molecules for epitopes from viral Ags (23–25). Of the five known gp100 epitopes, three bind to purified HLA-A*0201 molecules with intermediate affinity, and two bind with high affinity (19). Thus, at least for gp100 peptides with intermediate binding affinity, it seemed possible to enhance immunogenicity by making conservative modifications that increased HLA-A*0201-binding affinity.

By sequencing pooled peptides isolated from class I MHC molecules on a variety of cells, binding motifs have been defined that correlate high peptide-binding affinity to the predominance of certain amino acids at particular positions in peptide sequences (26–32). For example, for HLA-A*0201, leucine (L) and methionine

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[2] Abbreviations used in this paper: TIL, tumor-infiltrating lymphocytes; CM, complete medium.

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(M) are most frequently isolated at the second position from the amino terminus (P2), and valine (V) is predominant at P9. Each of the three common epitopes from gp100 contains a dominant residue at one, but not both, primary HLA-A*0201-binding anchor positions: $G9_{154}$ and $G9_{209}$ contain V at P9 but threonine (T) at P2, and $G9_{280}$ contains L at P2 but alanine (A) at P9. In previous studies using peptides from viral Ags, amino acid substitutions at primary and secondary class I MHC-binding anchor positions enhanced the immunogenicity of peptides against the native protein (33, 34). Therefore, although gp100 is a nonmutated self protein, it seemed possible that analogous amino acid substitutions might enhance the ability of peptides from this Ag to induce melanoma-reactive CTL.

In the current study, single and double amino acid substitutions were introduced into the three common epitopes from gp100 ($G9_{154}$, $G9_{209}$, and $G9_{280}$) at HLA-A*0201-binding anchor positions. Several modified gp100 peptides bound to HLA-A*0201 with greater affinity than unmodified peptides and were recognized by TIL specific for the native epitopes. With several modified gp100 peptides ($G9_{209}$ 2M, $G9_{209}$ 1F2L, and $G9_{280}$ 9V), melanoma-reactive CTL were induced from PBL of HLA-$A2^+$ melanoma patients in vitro more efficiently than with the natural peptides. These results demonstrate a relationship between immunogenicity and MHC-binding affinity for peptides from nonmutated self proteins. Furthermore, a new approach is suggested to improve the effectiveness of immunotherapy in patients with melanoma using modified peptides from tumor Ags.

Materials and Methods

Cultured cell lines

Melanoma-specific T cell lines were established from TIL, as previously described (10, 35). Several of these TIL recognize gp100 epitopes in the context of HLA-A2 (19–21) and were used in the work presented here: TIL 1200 (specific for $G9_{154}$), TIL 620 ($G9_{209}$), and TIL 660 and 1143 ($G9_{280}$).

Two melanoma lines, 624 mel (HLA-$A2^+$, gp100^+) and 397 mel (HLA-$A2^-$, gp100^+), were established in our laboratory (36); and clones of 624 mel, 624.38 (HLA-$A2^+$, gp100^+), and 624.28 (HLA-$A2^-$, gp100^+) were generated by a limiting dilution method (37). The melanoma line SKmel23 (HLA-$A2^+$, gp100^+) was kindly provided by Thierry Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). All tumor lines and T2 cells (HLA-$A2^+$ peptide transporter associated protein-deficient T-B cell hybrid) (38) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD).

Peptide synthesis and evaluation of binding affinity to HLA-A*0201

Three nonapeptides from gp100 were identified previously as common immunogenic epitopes recognized by TIL from several HLA-$A2^+$ patients with metastatic melanoma (19–21): $G9_{154}$ (KTWGQYWQV), $G9_{209}$ (ITDQVPFSV), and $G9_{280}$ (YLEPGPVTA). Single and double amino acid substitutions were made in each of these peptides according to the HLA-A*0201 binding motif (26–32) to enhance its binding affinity to class I MHC molecules without significantly altering recognition by TIL. Peptides were synthesized using a solid-phase method based on fluorenylmethoxycarbonyl (Fmoc) chemistry on a multiple peptide synthesizer (model AMS 422; Gilson Co., Worthington, OH), as previously described (39). Generally, purity was >85%, as evaluated by reverse-phase HPLC. Candidate peptides identified in an initial screening for recognition by TIL and CTL induction were further purified (>94%) by reverse-phase HPLC on a POROS 10 column (PerSeptive Biosystems, Cambridge, MA) using a 0.05% trifluoroacetic acid/water-acetonitrile gradient; the m.w. of each peptide was verified by laser desorption mass spectrometry (Bio-Synthesis, Lewisville, TX).

The relative binding affinity of each peptide to HLA-A*0201 was evaluated on the basis of the inhibition of binding of a standard radiolabeled peptide to purified MHC molecules, as previously described (40). Briefly, the test peptide at various concentrations (1 nM to 100 μM) was coincubated with 5 nM of iodinated peptide HBc_{18-27} (FLPSDYFPSV), soluble HLA-A*0201 heavy chain, and human β_2-microglobulin for 2 days at room temperature in the presence of protease inhibitors. The percentage of labeled peptide bound to HLA-A*0201 was determined by gel filtration; the concentration of test peptide necessary to inhibit the binding of the iodinated peptide by 50% (ID_{50}) was calculated. Peptide binding was defined as high (ID_{50} < 50 nM), intermediate (50 nM < ID_{50} < 500 nM), or weak (ID_{50} > 500 nM).

The relative binding affinity of each modified gp100 peptide compared with that of the respective unmodified (parent) peptide was expressed as a ratio (R):

$$R = \frac{ID_{50}\text{ (parent peptide)}}{ID_{50}\text{ (modified peptide)}}$$

R > 1 indicated that the modified peptide bound to HLA-A*0201 with greater affinity than the unmodified peptide; R < 1 indicated that the modified peptide bound with lower affinity.

In vitro induction of gp100-specific, melanoma-reactive CTL

To compare the immunogenicity of modified gp100 peptides with that of the native epitopes, peptide-specific CTL lines were generated as previously described (22, 37). Briefly, PBMC were isolated from the peripheral blood (buffy coats or leukapheresis samples) of HLA-$A2^-$ patients with metastatic melanoma by centrifugation on Ficoll-Hypaque gradients. HLA-A2 subtypes were determined by sequencing DNA from six of seven patients evaluated (all except patient 2), and all were HLA-A*0201.

CTL cultures were established initially by plating PBMC from cryopreserved samples in 24-well plates (1.5×10^6 cells/ml; 2 ml/well) in complete medium (CM; Iscove's modified DMEM with 25 mM HEPES, 10% heat-inactivated human AB serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 mcg/ml streptomycin; Biofluids; Sigma Chemical Co., St. Louis, MO; Pellfreez, Brown Deer, WI) in the presence of 1 μM of peptide. Two days later, 30 IU/ml of rIL-2 (Chiron Co., Emeryville, CA) was added to cultures. Lymphocytes were restimulated weekly using peptide-pulsed autologous PBMC as APC: 1) responder cells were harvested, and 2 to 3 $\times 10^6$ cells were replated in new 24-well plates (2.5×10^5 cells/ml; 2 ml/well) in CM; 2) autologous PBMC were thawed in CM with 30 U/ml of DNase (type I; Sigma Chemical Co.) to inhibit cell aggregation, washed once, resuspended in CM with 1 μM of peptide in 15-ml conical tubes ($1–3 \times 10^6$ cells/ml; 6–12 ml/tube), and incubated 3 to 4 h at 37°C; 3) these PBMC (stimulators) were irradiated with 3000 rad, washed once, and added to responder cells at responder to stimulator ratios between 1:4 and 1:12; and 4) 1 day later, 30 IU/ml of rIL-2 was added to cultures.

Bulk T cell cultures were evaluated for recognition of gp100 peptides and HLA-$A2^+$ melanoma cells ~7 days after restimulation, beginning at week 3 (after two restimulations). In addition, at each time point, responder T cells were cryopreserved for later studies to assess the kinetic development of Ag recognition. Frozen lymphocytes were thawed and cultured 24 to 48 h in 24-well plates (~10^6 cells/ml; 2 ml/well) in CM containing 30 or 300 IU/ml of rIL-2 before Ag-recognition studies.

At week 6 (after five restimulations), CD3, CD4, and CD8 expression by peptide-induced CTL was examined by fluorescence-activated flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA), as previously described (41).

Evaluation of Ag recognition by TIL and peptide-induced CTL

^{51}Cr release cytotoxicity assays and cytokine (IFN-γ) release assays were performed to evaluate the recognition of gp100 peptides and HLA-$A2^+$, gp100^+ melanoma cells (bulk SKmel23, bulk 624 mel, and/or clonal 624.38 mel) by peptide-induced CTL, as previously reported (10). All HLA-$A2^+$, gp100^+ melanoma cell lines were recognized similarly by gp100-reactive CTL. Briefly, ^{51}Cr-labeled T2 cells were preincubated with 1 μM of peptide ~1 h at 37°C; these cells and ^{51}Cr-labeled melanoma cells were then used as targets in cytotoxicity assays (5000 targets/well). Target and effector cells were coincubated for 4 h at 37°C, and the radioactivity in coculture supernatants was determined by gamma counting. The percent specific lysis of target cells by CTL was calculated, and spontaneous release never exceeded 25% of the maximum.

In cytokine release assays, 10^5 bulk CTL were coincubated with 10^5 tumor cells or peptide-loaded T2 cells for ~20 h at 37°C. The concentration of human IFN-γ in coculture supernatants was then determined using either a previously described ELISA (10) or a commercially available ELISA kit (Endogen, Cambridge, MA).

Table I. *Amino acids used for modification of gp100 epitopes*

	Position from N Terminus				
				Residues primarily involved in TCR recognition	
	1	2	3	4–8	9/10
Dominant primary anchor residues[a]		L M			V
Preferred or acceptable residues	F W Y A M L	I	F W Y A M S		L I

[a] Identified by sequencing pools of peptides eluted from HLA-A*0201 on cells (26–32).

Results

*Binding affinity of modified gp100 peptides to HLA-A*0201*

Previous reports have suggested that peptides from viral proteins that bind with high affinity to class I MHC molecules elicit stronger CTL immune responses than those that bind with lower affinity (23, 24). Therefore, to enhance the immunogenicity of peptides from the melanoma Ag gp100, amino acid substitutions were introduced into three previously identified epitopes (19–21) to enhance binding to HLA-A*0201 without significantly altering recognition of the peptide/MHC complex by an appropriate TCR (Table I) (26–32).

Each of the three common peptides from gp100 ($G9_{154}$, $G9_{209}$, and $G9_{280}$) is nine amino acids in length, and each contains a dominant residue at one, but not both, primary HLA-A*0201-binding anchor positions: $G9_{154}$ and $G9_{209}$ contain V at P9 but T at P2, and $G9_{280}$ contains L at P2 but A at P9. Therefore, amino acid substitutions were introduced initially into these gp100 epitopes at the primary anchor positions containing nondominant amino acids. Subsequently, single and double amino acid substitutions were introduced at the secondary anchor positions, P1 and P3, according to a previous study (28), alone or in conjunction with modifications at the primary anchor positions, P2 and P9. However, no substitutions were made at P4-P8 because these positions are believed to be directly involved in TCR contact with the MHC-peptide complex.

The binding affinity of each peptide to HLA-A*0201 was evaluated on the basis of the inhibition of binding of a standard radiolabeled peptide, HBc_{18-27} (FLPSDYFPSV), to purified MHC molecules (40). The concentration of peptide necessary to inhibit the binding of the standard peptide by 50% (ID_{50}) was calculated; peptide binding was defined as high ($ID_{50} < 50$ nM), intermediate (50 nM $< ID_{50} <$ 500 nM), or weak ($ID_{50} >$ 500 nM). The binding affinity of each modified gp100 peptide compared with that of the respective unmodified peptide was expressed as the ratio (R) of ID_{50} (parent peptide) to ID_{50} (modified peptide): $R > 1$ indicated that the modified peptide bound to HLA-A*0201 with greater affinity than the parent peptide.

The amino acid substitutions introduced into $G9_{154}$ were as follows: L, M, or I replaced T at P2 (2L, 2 M, 2I); W, F, Y, A, or L replaced K at P1 (1W, 1F, 1Y, 1A, 1L); Y or F replaced W at P3 (3Y, 3F); and L was substituted at P2 simultaneously with modifications at P1 (1W2L, 1F2L, 1Y2L, 1A2L, 1L2L). The native $G9_{154}$ peptide bound to HLA-A*0201 with high affinity ($ID_{50} = 5.7$ nM). Therefore, substitutions in this peptide at P1 and P2 only modestly enhanced class I MHC molecule binding (data not shown): the modified $G9_{154}$ peptide with the highest HLA-A*0201-binding affinity was $G9_{154}$ 1L2L, with ID_{50} equal to 1.6 nM.

Amino acid substitutions introduced into $G9_{209}$ and HLA-A*0201-binding affinities of the resulting peptides are presented in Table II. The binding affinity of the natural $G9_{209}$ peptide was within the intermediate range ($ID_{50} = 172$ nM), and most of the modified $G9_{209}$ peptides bound to HLA-A*0201, with significantly greater affinity than the naturally occurring epitope.

Modifications of $G9_{280}$ are presented in Table III. The binding affinity of this unmodified peptide was also within the intermediate range ($ID_{50} = 455$ nM); consequently, amino acid substitutions at primary and secondary anchor positions in $G9_{280}$ dramatically enhanced HLA-A*0201-binding affinity.

Recognition of modified peptides by TIL specific for native gp100 epitopes

Many modified gp100 peptides bound to HLA-A*0201 with greater affinity than the respective parent peptides. However, this did not imply that these modified peptides could be recognized by TCRs reactive with the natural gp100 epitopes that are likely to be expressed in the context of class I MHC molecules on the surfaces of melanoma cells. As an initial evaluation of cross-recognition between modified and unmodified peptides, IFN-γ secretion by TIL specific for the natural gp100 epitopes was measured in response to each substituted peptide at a concentration of 1 μM: $G9_{154}$ (data not shown), $G9_{209}$ (Table II), and $G9_{280}$ (Table III). Similar results were obtained from ^{51}Cr release cytotoxicity assays (data not shown). Within each group of peptides, several modifications resulted in loss of recognition by TIL specific for the natural epitope. However, several substituted peptides bound to HLA-A*0201 with higher affinity than the respective parent peptide and maintained recognition by appropriate TIL: $G9_{154}$ 2L, 2I, 1A, 1A2L (data not shown); $G9_{209}$ 2L, 2M, 2I, 1F, 1Y, 1W2L, 1F2L, 1Y2L (Table II); and $G9_{280}$ 9V, 9L, 9I, 1F (Table III). These peptides were selected as candidates to induce melanoma-reactive CTL in subsequent studies.

In vitro induction of melanoma-reactive CTL with modified gp100 peptides

The ability of candidate peptides described above to induce melanoma-reactive CTL specific for gp100 was evaluated in vitro by sensitizing PBL from HLA-$A2^+$ patients with metastatic melanoma. In addition, for each series of peptides, a few peptides were evaluated that had high binding affinity to HLA-A*0201 but were not recognized by TIL, since such modified peptides may also induce different T cells capable of recognizing the natural epitopes. In a preliminary study, a small panel of peptides was used to induce CTL from a single patient (patient 1), and a larger group of peptides was used to stimulate PBL from two additional patients (patients 2 and 3). Based on this initial peptide screening in three patients, several candidate peptides ($G9_{209}$ 2M and 1F2L; $G9_{280}$ 9V) were selected for CTL induction studies in four additional patients (patients 4–7) to evaluate the reproducibility of the preliminary findings.

Mononuclear cells were isolated from peripheral blood and cultured for 1 wk with 1 μM of peptide and 30 IU/ml of IL-2. Responder lymphocytes were restimulated weekly with peptide-pulsed autologous PBMC, as previously described (37). For each patient during a particular round of restimulation, the responder to stimulator ratio was constant within a group of peptides to ensure that the reactivity of CTL generated with modified peptides could be directly compared with that of CTL induced with the parent peptide. Within five rounds of restimulation, lymphocytes from all seven patients proliferated, although significant variations in

Table II. *Initial screening of modified $G9_{209}$ peptides for recognition by TIL and CTL induction*

$G9_{209}$ Peptide Modification	Sequence	HLA-A*0201 Binding Affinity[a] ID_{50}(parent)/ID_{50}(mod)	Recognition by TIL620[b] (1 μM peptide) IFN-γ (pg/ml)	Recognition of $G9_{209}$ by CTL Induced with Modified Peptides[c] (5 weeks in culture) Patient 1 % specific lysis (40:1 E:T)	Patient 2 IFN-γ[d] (pg/ml)	Patient 3 IFN-γ[d] (pg/ml)
Parent	ITDQVPFSV	1.0	2672	6 (0)[e]	2	346
2L	ITLDQVPFSV	52	2316	ND	145	46
2M	ITMDQVPFSV	9.0	2574	2 (0)	555	438
2I	ITIDQVPFSV	4.3	2266	10 (0)	4	19
1F	FTDQVPFSV	2.8	2541	ND	6	41
1W	WTDQVPFSV	0.24	1994	ND	0	0
1Y	YTDQVPFSV	2.0	2382	ND	0	31
3W	ITWQVPFSV	5.0	113	ND	ND	ND
3F	ITFQVPFSV	2.6	562	ND	ND	ND
3Y	ITYQVPFSV	5.2	257	ND	ND	ND
3A	ITAQVPFSV	1.8	120	ND	ND	ND
3M	ITMQVPFSV	4.3	2214	ND	ND	ND
3S	ITSQVPFSV	0.27	1212	ND	ND	ND
2L3W	ILWQVPFSV	102	329	ND	0	8
2L3F	ILFQVPFSV	86	243	ND	ND	ND
2L3Y	ILYQVPFSV	35	120	ND	ND	ND
2L3A	ILAQVPFSV	15	163	ND	ND	ND
2L3M	ILMQVPFSV	23	564	ND	ND	ND
2L3S	ILSQVPFSV	8.6	464	ND	ND	ND
1W2L	WLDQVPFSV	15	2359	ND	0	195
1F2L	FLDQVPFSV	79	2688	85 (0)	0	2
1Y2L	YLDQVPFSV	76	2445	ND	2	103

[a] Peptide binding affinity to HLA-A*0201 was evaluated by measuring the concentration of peptide necessary to inhibit the binding of a standard radiolabeled peptide by 50% (ID_{50}). The ratio (R) presented here indicates the relative HLA-A*0201 binding affinity of the modified peptide (mod) compared to the parent peptide (parent): R > 1 indicates the modified peptide bound with greater affinity than the parent peptide. ID_{50} ($G9_{209}$ parent) = 172 nM.
[b] IFN-γ release by TIL in the presence of T2 cells preincubated with 1 μM peptide. IFN-γ (T2 cells without exogenous peptide) = 454 pg/ml.
[c] Cytotoxicity or IFN-γ release by CTL in the presence of T2 cells preincubated with 1 μM peptide.
[d] IFN-γ release by CTL above the background of T2 cells without exogenous peptide.
[e] Values in parentheses indicate background % cytolysis of T2 cells without exogenous peptide.

growth were observed between cells originating from different patients: at week 6, bulk culture expansions ranged from ~50-fold (patient 2) to ~5×10^5-fold (patient 7) from the initial number of PBMC plated. No significant differences were observed in lymphocyte expansion with different peptides in a series of peptides from a single patient (data not shown).

Bulk CTL were evaluated for recognition of both modified and parent gp100 peptides and HLA-A2$^+$, gp100$^+$ melanoma cells ~7 days after restimulation, beginning at week 3 (after two restimulations). In addition, in patients 4–7, the kinetic development of Ag recognition by peptide-induced CTL was assessed over the entire course of the 6-wk sensitization: within a given series of peptides, cryopreserved CTL samples from a single patient after each round of restimulation were thawed and evaluated simultaneously for recognition of gp100 peptides and HLA-A2$^+$ melanoma cells. For each CTL culture, both ^{51}Cr release cytotoxicity and cytokine (IFN-γ) release assays were performed using peptide-loaded T2 cells and tumors as targets or stimulators; data from both types of assays were similar. In these experiments, evaluation of melanoma recognition by CTL was essential to determine whether modified peptides could induce T cells with high affinity TCRs capable of recognizing the native epitopes that are likely to be expressed at low density on the surfaces of tumor cells. In general, greater CTL reactivity was observed in response to T2 cells pulsed with peptides compared with melanoma cells, both in cytolytic and cytokine release assays. This may have been primarily due to low expression of gp100 epitopes on tumor cells or other factors, such as lower sensitivity of melanoma cells to lysis by CTL and heterogeneity in bulk melanoma cultures.

The efficiency of melanoma-reactive CTL induction was compared between modified and unmodified peptides based on the following: 1) overall levels of specific cytotoxicity and IFN-γ release by CTL in response to peptide-loaded T2 cells and HLA-A2$^+$, gp100$^+$ melanoma cells; and 2) number of restimulations (time) required for specific Ag recognition.

$G9_{154}$ peptides

In an initial screening of CTL induction with modified $G9_{154}$ peptides, the following candidate peptides were chosen based on high binding affinity to HLA-A*0201 and/or recognition by $G9_{154}$-reactive TIL: $G9_{154}$ 2L, 2M, 2I, 1F, 1A, 3Y, 1A2L, 1L2L, and 1F2L. As evaluated by cytotoxicity and cytokine release by bulk CTL from three patients after four rounds of restimulation in the presence of peptide-loaded T2 cells, no modified $G9_{154}$ peptide consistently induced $G9_{154}$-reactive CTL more efficiently than the parent peptide (data not shown). In addition, because the parent $G9_{154}$ peptide bound to HLA-A*0201 with high affinity (ID_{50} = 5.7 nM) and modified $G9_{154}$ peptides only demonstrated a 1- to 3.6-fold increase in HLA-A*0201-binding affinity, this gp100 epitope was not pursued further.

$G9_{209}$ peptides

In contrast to $G9_{154}$ peptides, results from initial CTL induction studies in three patients with modified $G9_{209}$ peptides demonstrated that CTL reactive against the native gp100 epitope could be induced more efficiently with several substituted peptides than with the natural peptide itself (Table II). In patient 1, within four restimulations, CTL specific for $G9_{209}$ were induced with $G9_{209}$ 1F2L, but not with the parent peptide; in patient 2, only $G9_{209}$ 2M efficiently induced CTL that recognized the natural epitope; and in patient 3, both the unmodified peptide and $G9_{209}$ 2M efficiently

Table III. *Initial screening of modified $G9_{209}$ peptides for recognition by TIL and CTL induction*

$G9_{280}$ Peptide Modification	Sequence	HLA-A*0201 Binding Affinity[a] ID_{50}(parent) / ID_{50}(mod)	Recognition by TIL1143[b] (1 μM peptide) IFN-γ (pg/ml)	Recognition of $G9_{280}$ by CTL Sensitized with Modified Peptides[c] (5 w in culture) Patient 1 % specific lysis (40:1 E:T)	Patient 2 IFN-γ[d] (pg/ml)	Patient 3 IFN-γ[d] (pg/ml)
Parent	YLEPGPVTA	1.0	641	11 (3)[e]	0	47
9V	YLEPGPVTV	10	599	87 (0)	210	654
9L	YLEPGPVTL	5.2	487	ND	16	156
9I	YLEPGPVTI	7.0	610	ND	61	185
1F	FLEPGPVTA	3.6	286	ND	0	156
1W	WLEPGPVTA	0.55	275	ND	0	19
3Y	YLYPGPVTA	27	59	ND	ND	ND
3W	YLWPGPVTA	141	160	ND	0	5
3F	YLFPGPVTA	141	192	ND	ND	ND
3M	YLMPGPVTA	105	262	ND	ND	ND
3S	YLSPGPVTA	11	104	ND	ND	ND
3A	YLAPGPVTA	49	120	ND	ND	ND
3M9V	YLMPGPVTV	39	46	ND	ND	ND
3S9V	YLSPGPVTV	20	89	ND	ND	ND
3A9V	YLAPGPVTV	30	25	ND	ND	ND
3Y9V	YLYPGPVTV	51	27	ND	ND	ND
3F9V	YLFPGPVTV	79	48	ND	ND	ND
3W9V	YLWPGPVTV	61	250	ND	ND	ND

[a] Peptide binding affinity to HLA-A*0201 was evaluated by measuring the concentration of peptide necessary to inhibit the binding of a standard radiolabeled peptide by 50% (ID_{50}). The ratio (R) presented here indicates the relative HLA-A*0201 binding affinity of the modified peptide (mod) compared to the parent peptide (parent): R > 1 indicates the modified peptide bound with greater affinity than the parent peptide. (ID_{50} ($G9_{280}$ parent) = 455 nM.

[b] IFN-γ release by TIL in the presence of T2 cells preincubated with 1 μM peptide. IFN-γ (T2 cells without exogenous peptide) = 89 pg/ml.

[c] Cytotoxicity or IFN-γ release by CTL in the presence of T2 cells preincubated with 1 μM peptide.

[d] IFN-γ release by CTL above the background of T2 cells without exogenous peptide.

[e] Values in parentheses indicate background percentage of cytolysis of T2 cells without exogenous peptide.

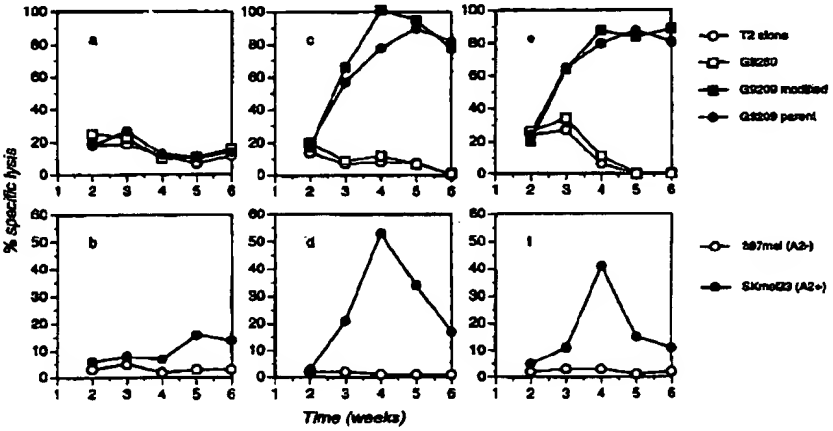

FIGURE 1. Kinetic development of Ag recognition in CTL from patient 6 induced with $G9_{209}$ peptides. CTL were induced by weekly stimulation with autologous PBMC pulsed with modified $G9_{209}$ peptides. Each week beginning at week 2, recognition of modified and natural peptides and of HLA-$A2^+$ melanoma cells by CTL was evaluated using peptide-loaded T2 cells and SKmel23 cells as targets in ^{51}Cr release cytotoxicity assays 20:1 E:T. Graphs present the following information: peptide (*a*) and melanoma (*b*) recognition by CTL induced with $G9_{209}$ parent; peptide (*c*) and melanoma (*d*) recognition by CTL induced with $G9_{209}$ 2M; and peptide (*e*) and melanoma (*f*) recognition by CTL induced with $G9_{209}$ 1F2L. In *a*, *c*, and *e*, T2 alone (○) indicates lysis of T2 cells without exogenous peptide, and $G9_{280}$ (□) indicates lysis of T2 cells pulsed with $G9_{280}$ parent as negative controls. In *c*, $G9_{209}$ modified (■) indicates lysis of T2 cells pulsed with $G9_{209}$ 2 M; and in *e*, $G9_{209}$ modified (■) indicates lysis of T2 cells pulsed with $G9_{209}$ 1F2L. In *b*, *d*, and *f*, (○) indicates lysis of 397 mel HLA-$A2^-$ as a negative control.

induced $G9_{209}$-reactive CTL. ^{51}Cr release cytotoxicity assays performed with peptide-induced CTL from patients 2 and 3 showed similar trends of peptide recognition as those seen in cytokine release assays (data not shown). Based on these results, $G9_{209}$ 2M and $G9_{209}$ 1F2L were selected for further evaluation in patients 4 through 7.

One example of the recognition of $G9_{209}$ peptides and HLA-$A2^+$ melanoma cells by CTL induced with modified $G9_{209}$ peptides is presented in Figure 1. These data are shown as CTL reactivities in bulk T cell cultures, and therefore, may not reflect specific activity per $CD8^+$ cell. Within five rounds of restimulation (6 wk in culture), it was not possible to induce peptide-specific, melanoma-reactive CTL from patient 6 using the unmodified $G9_{209}$ peptide (Fig. 1, *a* and *b*). However, after two restimulations, CTL from this patient induced with $G9_{209}$ 2M (Fig. 1*c*) and $G9_{209}$ 1F2L (Fig. 1*e*) were clearly specific for both the modified peptide with which they were generated and the unmodified peptide, $G9_{209}$. Furthermore, after four restimulations, these CTL were

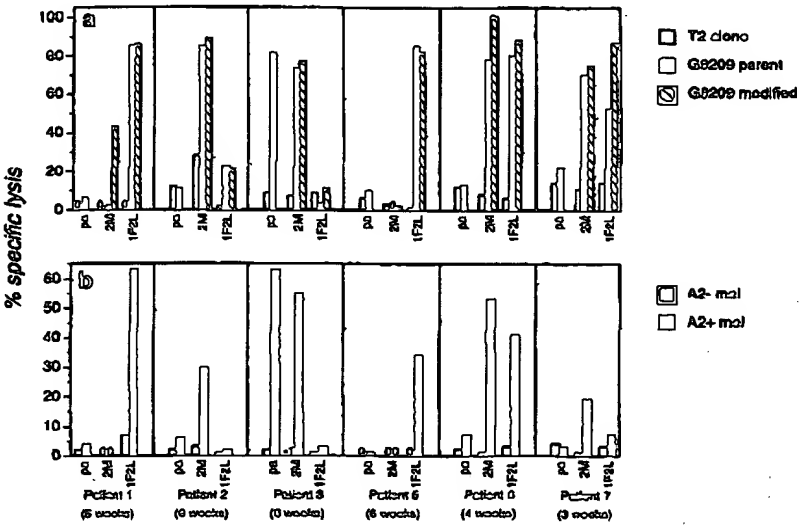

FIGURE 2. Melanoma and peptide recognition by CTL induced with $G9_{209}$ peptides. CTL were induced by weekly stimulation with autologous PBMC pulsed with modified $G9_{209}$ peptides. Each week, recognition of modified and natural peptides and of HLA-A2$^+$ melanoma cells by CTL was evaluated using peptide-loaded T2 cells and either 624.38 mel or SKmel23 cells, which behave similarly, as targets in ^{51}Cr release cytotoxicity assays 20:1 E:T. The data shown indicates target lysis at the earliest time point at which specific melanoma reactivity was observed by CTL from each patient. No data is presented for patient 4 because no melanoma-reactive CTL were induced from this patient with any of the $G9_{209}$ peptides. In *a*, T2 alone indicates lysis of T2 cells without exogenous peptide as a negative control; $G9_{209}$ modified indicates lysis of T2 cells pulsed with the peptide used to stimulate those T cells, i.e., $G9_{209}$ 2M for CTL induced with $G9_{209}$ 2M, and $G9_{209}$ 1F2L for CTL induced with $G9_{209}$ 1F2L. In *b*, A2$^-$ mel indicates lysis of either 624.28 mel or 397 mel HLA-A2$^-$, which behave similarly, as a negative control. PA represents CTL induced with $G9_{209}$ parent; 2M represents CTL induced with $G9_{209}$ 2M; and 1F2L represents CTL induced with $G9_{209}$ 1F2L.

highly reactive against an HLA-A2$^+$ melanoma cell line (SKmel23), but not against an HLA-A2$^-$ melanoma line (397 mel) (Fig. 1, *d* and *f*). It is currently unclear why CTL reactivity, particularly to melanoma cells, occasionally decreased after multiple peptide stimulations (e.g., Fig. 1, *c*, *d*, and *f*); however, it may simply reflect a need to optimize the in vitro culture protocol.

Cumulative results from CTL induction studies in all seven patients with $G9_{209}$ peptides are presented in Figure 2. From all patients, except patient 4, CTL could be induced that recognized both the natural $G9_{209}$ peptide and HLA-A2$^+$, gp100$^+$ melanoma cells. Within five rounds of restimulation with the parent $G9_{209}$ peptide, melanoma-reactive CTL were induced from only two of seven patients evaluated: patients 3 and 7 after five restimulations. However, tumor-specific CTL were generated in four patients (patients 2, 3, 6, and 7) with $G9_{209}$ 2M and three patients (patients 1, 5, and 6) with $G9_{209}$ 1F2L (Fig. 2). In addition, in patient 7, from whom melanoma-reactive CTL were induced with both $G9_{209}$ parent and $G9_{209}$ 2M, specific tumor reactivity was apparent after two restimulations with the modified peptide (Fig. 2) compared with five restimulations required for the natural peptide (data not shown).

In patient 1 with $G9_{209}$ 2M and in patient 7 with $G9_{209}$ 1F2L, CTL were induced that predominantly recognized the modified peptide used for sensitization (Fig. 2). This suggested that some bulk CTL cultures induced with modified $G9_{209}$ peptides may have contained populations of T cells not crossreactive between the modified and parent peptides. However, in all but these two cases, when peptide-specific CTL were induced with modified $G9_{209}$ peptides, CTL were crossreactive since melanoma reactivity was also observed (Fig. 2).

After five rounds of restimulation, CD3, CD4, and CD8 expression on CTL from patients 4 through 7 induced with $G9_{209}$ peptides was examined by fluorescence-activated flow cytometry. In all cases, >99% of viable cells were CD3$^+$ T cells. In patients 5, 6, and 7, from whom peptide-specific CTL were induced with at least one $G9_{209}$ peptide, the percentages of CD8$^+$ cells and corresponding reactivities after five restimulations were as follows: 1) T cells from patient 5 stimulated with $G9_{209}$ parent (45% CD8$^+$) or $G9_{209}$ 2M (94% CD8$^+$) demonstrated no reactivity, while those sensitized with $G9_{209}$ 1F2L (74% CD8$^+$) recognized both peptide and melanoma (Fig. 2); 2) T cells from patient 6 stimulated with $G9_{209}$ parent (45% CD8$^+$) were nonreactive, while those induced with $G9_{209}$ 2M (83% CD8$^+$) or $G9_{209}$ 1F2L (65% CD8$^+$) recognized both peptide and melanoma (Fig. 1); and 3) T cells from patient 7 sensitized with $G9_{209}$ parent (69% CD8$^+$) or $G9_{209}$ 2M (69% CD8$^+$) were peptide and melanoma reactive, but those stimulated with $G9_{209}$ 1F2L (23% CD8$^+$) were not (data not shown). Therefore, in these three patients it is unlikely that differences in recognition of $G9_{209}$ and melanoma between bulk CTL induced with different $G9_{209}$ peptides were solely due to differences in the percentages of CD8$^+$ cells. In addition, bulk T cell expansions did not vary significantly between modified and parent peptides, so that more melanoma-reactive CTL were induced in cultures sensitized with $G9_{209}$ 2M or $G9_{209}$ 1F2L compared with the natural epitope.

The mechanisms responsible for differences in the abilities of $G9_{209}$ 2M and $G9_{209}$ 1F2L to induce melanoma-reactive CTL from different patients are not clear; however, such differences may reflect diversity in the functional T cell repertoires of these patients or other factors not yet completely understood.

$G9_{280}$ peptides

Initial CTL induction studies in three patients with modified $G9_{280}$ peptides clearly demonstrated that CTL reactive against the native gp100 epitope were induced most efficiently in all three patients with the modified peptide $G9_{280}$ 9V (Table III). ^{51}Cr release cytotoxicity assays performed with peptide-induced CTL from patients 2 and 3 showed similar trends of peptide recognition as those seen in cytokine release assays (data not shown). Based on these results, $G9_{280}$ 9V was further evaluated using PBL from patients 4 through 7.

One example of the recognition of $G9_{280}$ peptides and HLA-A2$^+$ melanoma cells by CTL induced with $G9_{280}$ 9V is presented

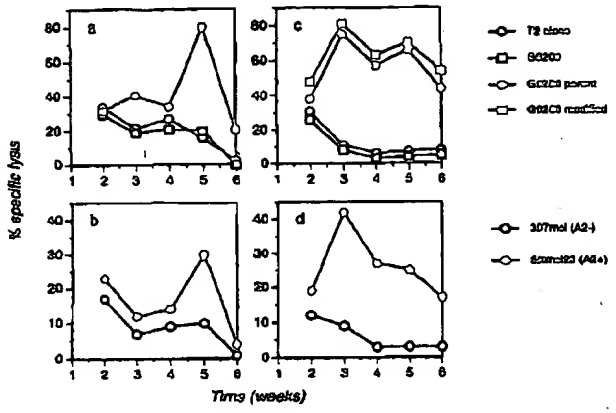

FIGURE 3. Kinetic development of Ag recognition in CTL from patient 7 induced with $G9_{280}$ peptides. CTL were induced by weekly stimulation with autologous PBMC pulsed with modified $G9_{280}$ peptides. Each week beginning at week 2, recognition of modified and natural peptides and of HLA-A2$^+$ melanoma cells by CTL was evaluated using peptide-loaded T2 cells and SKmel23 cells as targets in ^{51}Cr release cytotoxicity assays 20:1 E:T. Graphs present the following information: peptide (*a*) and melanoma (*b*) recognition by CTL induced with $G9_{280}$ parent; peptide (*c*) and melanoma (*d*) recognition by CTL induced with $G9_{280}$ 9V. In *a* and *c*, T2 alone (○) indicates lysis of T2 cells without exogenous peptide, and $G9_{209}$ (□) indicates lysis of T2 cells pulsed with $G9_{209}$ parent as negative controls. In *c*, $G9_{280}$ modified (□) indicates lysis of T2 cells pulsed with $G9_{280}$ 9V. In *b* and *d*, (○) indicates lysis of 397 mel HLA-A2$^-$ as a negative control.

in Figure 3. From patient 7, it was possible to induce peptide-specific, melanoma-reactive CTL with the unmodified $G9_{280}$ peptide (Fig. 3, *a* and *b*); however, specific peptide recognition and HLA-A2$^+$ melanoma reactivity were not observed before four rounds of restimulation. On the other hand, CTL induced with $G9_{280}$ 9V specifically recognized both $G9_{280}$-loaded T2 cells and HLA-A2$^+$ melanoma cells after two restimulations, 2 wk before specific reactivity was observed by CTL induced with the parent peptide (Fig. 3, *c* and *d*).

Cumulative results from CTL induction studies in all seven patients with $G9_{280}$ peptides are presented in Figure 4. From all patients except patient 5, CTL could be induced that recognized both the natural $G9_{280}$ peptide and HLA-A2$^+$, gp100$^+$ melanoma cells. Within five rounds of restimulation with the unmodified $G9_{280}$ peptide, melanoma-reactive CTL were induced from only one of seven patients evaluated (patient 7 after four restimulations). However, tumor-specific CTL were generated from six of seven patients (all except patient 5) with $G9_{280}$ 9V (Fig. 4).

After five rounds of restimulation, CD3, CD4, and CD8 expression on CTL from patients 4 through 7 induced with $G9_{280}$ peptides was examined by fluorescence-activated flow cytometry. In all cases, >99% of viable cells were CD3$^+$ T cells. In patients 4, 6, and 7, from whom peptide-specific CTL were induced with $G9_{280}$ 9V, the percentages of CD8$^+$ cells and corresponding reactivities after five restimulations were as follows: 1) T cells from patient 4 stimulated with $G9_{280}$ parent (14% CD8$^+$) were nonreactive, while those induced with $G9_{280}$ 9V (94% CD8$^+$) recognized both peptide and melanoma (Fig. 4); 2) T cells from patient 6 stimulated with $G9_{280}$ parent (71% CD8$^+$) were also nonreactive, while those induced with $G9_{280}$ 9V (93% CD8$^+$) recognized peptide and melanoma (Fig. 4); and 3) T cells from patient 7 stimulated with $G9_{280}$ parent (14% CD8$^+$) reacted weakly with peptide, while those induced with $G9_{280}$ 9V (38% CD8$^+$) were more clearly peptide specific (Fig. 3). Therefore, in patients 4 and 7, the increased numbers of CD8$^+$ T cells in CTL induced with $G9_{280}$ 9V may have been related to the enhanced melanoma reactivities of these bulk cultures. However, in patient 6, it is unlikely that differences in CD8 expression could solely account for the observed difference in melanoma reactivity between bulk CTL induced with $G9_{280}$ 9V and those stimulated with the unmodified peptide. Therefore, $G9_{280}$ 9V consistently enhanced the induction of melanoma-reactive CTL in either CD8$^+$ T cell subsets or bulk T cell cultures more efficiently than the natural peptide. In addition, bulk T cell expansions did not vary significantly between the modified and parent peptides, so that more melanoma-reactive CTL were induced in cultures sensitized with $G9_{280}$ 9V compared with the natural epitope.

Discussion

Three common epitopes from the melanoma Ag gp100 have been identified that are recognized by HLA-A2-restricted TIL derived from different patients with metastatic melanoma (19–21): $G9_{154}$ (KTWGQYWQV), $G9_{209}$ (ITDQVPFSV), and $G9_{280}$ (YLEPGPV TA). Furthermore, recognition of these epitopes by T cells in vitro has been correlated with in vivo tumor regression after adoptive transfer of TIL, suggesting that gp100 may be a potent tumor rejection Ag (19). In a previous study, CTL responses to gp100 epitopes were evaluated in vitro by using these peptides to sensitize T cells from PBL of HLA-A2$^+$ melanoma patients (22). With a given gp100 peptide, melanoma-reactive CTL could only be induced in a limited number of patients, and numerous rounds of restimulation were required. This finding prompted the current investigation aimed at enhancing the immunogenicity of gp100 peptides.

Upon immunization with a foreign protein, T cell responses are generally induced against a limited number of epitopes (dominant determinants). This may result because only a few peptides from a given protein are expressed at high levels in the context of MHC molecules on the surfaces of infected cells (42, 43). One major factor influencing peptide expression on the APC surface is the binding affinity of the peptide to MHC molecules; and some studies have suggested that immunodominance of foreign peptides in CTL responses is correlated with high peptide-binding affinity to appropriate MHC restriction elements (25). In fact, most previously identified epitopes from viral Ags recognized in the context of HLA-A*0201 bound to this MHC molecule with high affinity and contained dominant amino acids at primary MHC-binding anchor positions (L or M at P2, and V or L at P9).

In contrast to foreign peptides, when self peptides are expressed at high density on a cell surface due to high MHC-binding affinity and/or effective Ag processing, tolerance seems to be induced, and reactive T cells are eliminated or inactivated (42–47). Therefore, self peptides that bind to MHC molecules with high affinity may be less likely to be immunogenic than foreign peptides with high binding affinity. In fact, immunodominant epitopes in CTL responses to self proteins may frequently be subdominant or cryptic, rather than dominant, determinants (44–47). This may explain the observation that many epitopes from melanoma Ags that are nonmutated self proteins, such as gp100 and MART-1, have relatively low binding affinities to HLA-A2 (46, 47).

A correlation has been demonstrated previously between immunogenicity of peptides and class I MHC-binding affinity (23, 24). This suggests that induction of melanoma-reactive CTL using gp100 peptides may be inefficient due to the relatively low binding affinities of these peptides to HLA-A*0201. However, recognition of these epitopes by TIL in vitro correlated with tumor regression

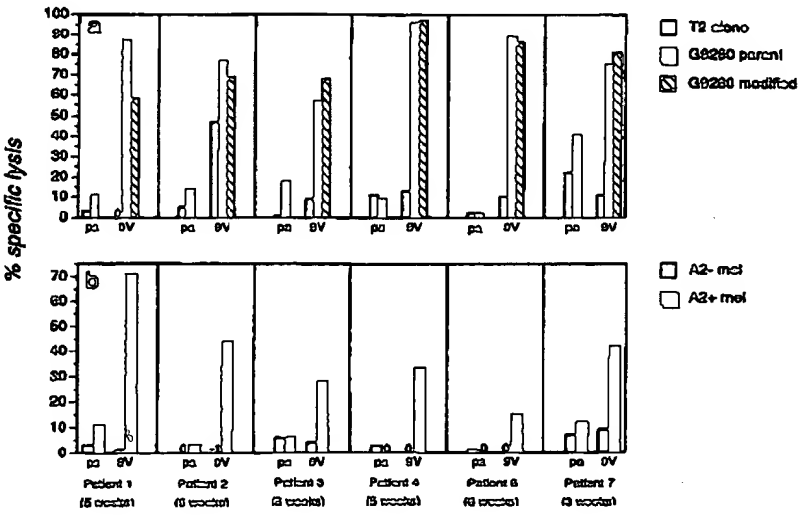

FIGURE 4. Melanoma and peptide recognition by CTL induced with $G9_{280}$ peptides. CTL were induced by weekly stimulation with autologous PBMC pulsed with modified $G9_{280}$ peptides. Each week, recognition of modified and natural peptides and of HLA-A2$^+$ melanoma cells by CTL was evaluated using peptide-loaded T2 cells and either 624.38 mel or SKmel23 cells, which behave similarly, as targets in ^{51}Cr release cytotoxicity assays 20:1 E:T. The data shown indicates target lysis at the earliest time point at which specific melanoma reactivity was observed by CTL from each patient. No data is presented for patient 5 because no melanoma-reactive CTL were induced from this patient with any of the $G9_{280}$ peptides. In *a*, T2 alone indicates lysis of T2 cells without exogenous peptide as a negative control; $G9_{280}$ modified indicates lysis of T2 cells pulsed with $G9_{280}$ 9V. In *b*, A2$^-$ mel indicates lysis of 624.28 mel or 397 mel, which behave similarly, as a negative control. PA represents CTL induced with $G9_{280}$ parent; and 9V represents CTL induced with $G9_{280}$ 9V.

in vivo (19). Therefore, protocols for inducing CTL responses to epitopes with low class I MHC-binding affinity may enable the development of effective melanoma therapies based on tumor Ags that are nonmutated self proteins. Such peptides are usually expressed at low density on the surfaces of APC and, therefore, are likely not to be presented efficiently to potentially reactive T cell precursors; i.e., peptides with low class I MHC-binding affinities may not be potent immunogens. The present investigation suggests a method by which the expression efficiency of such peptides may be enhanced: peptides can be modified in such a way as to increase their binding affinities to MHC molecules without significantly altering TCR recognition of the peptide/MHC complex.

In this study, single and double amino acid substitutions were introduced into the three common epitopes of gp100 ($G9_{154}$, $G9_{209}$, and $G9_{280}$) according to the HLA-A*0201 binding motif (Table I) (26–32). As an additional constraint, modifications were only made at positions not directly involved with TCR binding so that the resulting peptide would maintain recognition by TIL specific for the native epitope. As anticipated, many of the modified peptides bound with much greater affinity to HLA-A*0201 than the parent peptides (Tables II and III). However, many substitutions resulted in loss of recognition by gp100-reactive TIL, probably because conformational changes destroyed the TCR binding structure of the peptide/MHC complex.

Several modified gp100 peptides were identified that bound with high affinity to HLA-A*0201 and were recognized by gp100-reactive TIL. The ability of these peptides to induce melanoma-reactive CTL was then evaluated in vitro by stimulating PBL from HLA-A2$^+$ melanoma patients with peptide-pulsed autologous PBMC. Comparisons of induction efficiency between modified and natural epitopes were based on the potency of melanoma reactivity and the time required for the development of specific Ag recognition.

After five weekly restimulations with modified $G9_{154}$ peptides, it was not clear that any substituted peptide could consistently induce gp100-reactive CTL more efficiently than the parent peptide. Based on the observation that native $G9_{154}$ binds to HLA-A*0201 with high affinity, amino acid substitutions resulting in small increases in binding affinity may not be able to augment CTL induction efficiency. Alternatively, significant T cell tolerance may be induced to this peptide in some patients, making it difficult to elicit $G9_{154}$-reactive CTL, even under ideal conditions.

In contrast to $G9_{154}$, $G9_{209}$ binds to HLA-A*0201 with relatively low affinity (intermediate); and melanoma-reactive CTL were induced more efficiently with substituted peptides based on this epitope than with the natural peptide (Fig. 2). Within five rounds of restimulation with $G9_{209}$ parent, melanoma-reactive CTL were induced from only two of seven patients evaluated. However, tumor-specific CTL were generated in four patients with $G9_{209}$ 2M, and three patients with $G9_{209}$ 1F2L. In addition, in one patient from whom melanoma-reactive CTL were induced with the natural $G9_{209}$ peptide, specific tumor reactivity was apparent after fewer restimulations using the modified peptide, $G9_{209}$ 2M. Differences in the CTL induction capacity between $G9_{209}$ 2M and $G9_{209}$ 1F2L among patients may reflect differences in their functional T cell repertoires.

Like $G9_{209}$, $G9_{280}$ binds to HLA-A*0201 with relatively low affinity, and melanoma-reactive CTL were induced consistently with a modified version of this peptide ($G9_{209}$ 9V) more efficiently than with the natural peptide. Within five rounds of restimulation with $G9_{280}$ parent, melanoma-reactive CTL were induced from only one of seven patients evaluated; however, tumor-specific CTL were generated in six of seven patients with $G9_{280}$ 9V. Furthermore, in one patient, from whom melanoma-reactive CTL were induced with the unmodified $G9_{280}$ peptide, specific tumor reactivity was apparent after fewer restimulations with $G9_{280}$ 9V. In some patients (e.g., 4 and 7), significantly more CD8$^+$ T cells were present in bulk CTL cultures induced with $G9_{280}$ 9V compared with the unmodified peptide; such increases in the numbers of CD8$^+$ T cells may have been related to the increases in melanoma-reactive CTL in these cultures. However, in at least one patient (i.e., patient 6), both $G9_{280}$ and $G9_{280}$ 9V induced bulk CTL cultures containing many CD8$^+$ T cells (i.e., >70%), and yet, melanoma-reactive CTL were present only in cultures stimulated with the modified peptide.

Similar modifications were also introduced into the immunodominant epitope from the HLA-A2-restricted melanoma Ag, MART-1 ($M9_{27}$), to enhance class I MHC-binding affinity (data not shown). A few modified $M9_{27}$ peptides bound with high affinity to HLA-A*0201 and were recognized by MART-1-specific

TIL. However, when these peptides were used to seasitize T cells from HLA-A2$^+$ melanoma patients, none of the modified peptides consistently induced melanoma-reactive CTL more efficiently than the parent peptide. This may have resulted simply because these modified peptides were extremely insoluble in aqueous solution. Alternatively, because native $M9_{27}$ is highly immunogenic in vitro (37), it may not be possible to observe differences in CTL induction efficiency using these techniques.

In previous investigations, amino acid substitutions have been introduced into foreign peptides from viral Ags to enhance immunogenicity (33, 34). For example, in a murine model system, a natural peptide from the human papilloma virus protein E6 could not induce peptide-specific CTL; however, when a single amino acid substitution was made according to the H2-K^b binding motif, CTL were induced that recognized both the natural peptide and cells expressing the native E6 protein (33). A similar phenomena was observed using modified peptides based on the HLA-A2-restricted epitope from HIV-1 reverse transcriptase to sensitize lymphocytes from HIV$^+$, HLA-A2$^+$ patients in vitro (34). In the current study, amino acid substitutions in peptides from gp100 enhanced the capacity of these peptides to induce melanoma-reactive CTL. These results demonstrate that this approach may be useful to improve the immunogenicity of self peptides from human tumor Ags for immunotherapy. In fact, clinical protocols, in which patients with metastatic melanoma are being immunized with $G9_{209}$ 2M and $G9_{280}$ 9V in IFA, are in progress in the Surgery Branch at the National Cancer Institute (Bethesda, MD).

Other approaches for inducing CTL responses to epitopes expressed at low density on the surfaces of cells are currently being investigated. For example, recombinant viruses containing tumor Ag genes may allow for high levels of peptide expression resulting from intracellular production of large amounts of proteins (48, 49). Alternatively, epitopes may be attached covalently to MHC molecules on the surface of APC (50), allowing for long-term, high level expression of peptides that bind to MHC molecules with low affinity or are not processed efficiently (X. Kang, manuscript in preparation).

Most melanoma-reactive TIL recognize cultured melanocytes as well as melanoma cells in vitro. In addition, in a previous study, a significant correlation was observed between permanent depigmentation of the skin, suggesting destruction of melanocytes, and tumor regression in melanoma patients receiving IL-2-based immunotherapy (S. A. Rosenberg, manuscript submitted). These observations suggest that autoreactive T cells against nonmutated self epitopes may be involved in tumor regression in vivo in some instances. Therefore, one potential limitation to the use of modified gp100 peptides in the treatment of patients with melanoma may be the more frequent development of autoimmune disorders: although modified immunogens may mediate tumor regression more effectively, they may also induce strong reactivities against melanocytes.

The results presented in this work demonstrate that peptides modified at MHC-binding anchor positions can efficiently induce T cells specific for immunodominant epitopes from the melanoma Ag gp100, which may be cryptic self determinants. These techniques may be useful for studying immunologic phenomena such as molecular mimicry of viral and bacterial peptides in the initiation of T cell responses in autoimmune diseases (42, 43). These results also suggest means for improving current strategies in immunotherapy for patients with melanoma. For example, modified gp100 peptides could be used for active immunization or to induce melanoma-reactive CTL in vitro for adoptive transfer into patients. Alternatively, point mutations at MHC-binding anchor positions in epitopes from tumor Ags could be introduced into genes encoding these proteins for use in gene therapy protocols.

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